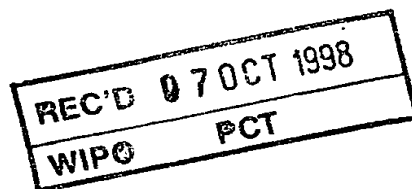


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- (71) Sökande                      *Vlaamse Instelling voor Technologisch Onderzoek,*  
 Applicant (s)                *Mol BE*  
                                      *School of Biological Sciences, Birmingham GB*  
                                      *Bo Mattiasson, Lund SE*  
                                      *Elisabeth Csöregi, Lund SE*  
                                      *Ibolya Bontidean, Lund SE*  
                                      *Gillis Johansson, Lund SE*  
                                      *Christine Berggren, Lund SE*

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*Evy Morin*  
 Evy Morin

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## PRIORITY DOCUMENT

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## Metal ion specific capacity affinity sensor

There is a need to measure trace concentration of heavy metal ions in the environment, in medicine, in food and other products. Heavy metals are toxic elements and it is therefore important to be able to determine them on the trace level. Some heavy metals, e.g. copper and zinc, are essential for the living cells. Various classical methods such as atomic absorption spectroscopy, inductively coupled plasma emission spectrometry and plasma emission mass spectrometry are in wide use. These methods require sophisticated instrumentation and skilled personnel. Therefore, there is a need for easier and cheaper methods. Electrochemical methods for metal ion determination include ion selective electrodes, polarography and other voltammetric electrodes. Biosensors are selective and sensitive analytical devices and several different biosensor configurations have been described in the past for heavy metal detection. Whole cell-based biosensors represent one possible design and these can utilize bacteria, yeasts, fungi, lichens, mosses and water plants as recognition element [Wittman, C.; Riedel, K.; Schmid, R. D. *Handb. Biosens. Electron. Noses*, 1997 299-332]. Another approach is to use enzymes for the detection. So far the use of apoenzymes has been the most successful and widely used method [Mattiasson, B.; Nilsson, H.; Olsson, B. *J. Appl. Biochem.*, 1 1979 377-384]. However, these sensors are characterized by limited selectivity and fairly low sensitivity.

Protein engineering has opened up the possibility to design and produce new proteins with e. g. higher selectivity than natural ones. The fusion protein GST-SmtA (SEQ.ID.NO.1) has been developed by protein engineering and it is reported to show a broad selectivity towards various heavy metal ions ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Hg}^{2+}$ ) [Shi, J.; Lindsay, W. P.; Huckle, J. W.; Morby, A. P.; Robinson, N. J. *FEBS*, 303, 1992 159-163]. Another fusion protein called MerR (SEQ.ID.NO.2) is engineered to be exclusively selective towards  $\text{Hg}^{2+}$  [Frantz, B.; O'Hallaran, T. V.; *Biochemistry*,

29, 1990 4747-4751]. A third protein, PbrR (SEQ.ID.NO.3) from the strain  
Alcaligenes eutrophus CH34 (The strain is deposited BCCM under the accession  
number LMG P-18077) is selective towards  $Pb^{2+}$ . A fourth protein, MerP  
(SEQ.ID.NO.4), is selective towards  $Hg^{2+}$ . It is believed that a large conformational  
5 change is taking place when heavy metal ions bind to these proteins. This invention  
describes a capacitive sensor which can detect these conformational changes  
directly.

Self-assembled monolayers of thiols, sulfides and disulfides on gold electrodes have  
10 been widely studied and long-chain alkanethiols are known to form insulating well-  
organized structures on gold substrates [Porter, M. D.; Bright, T. B.; Allara, D. L.;  
Chidsey, C. E. D. *J. Am. Chem. Soc.* 1987, 109, 3559-3568]. The binding formed  
between the sulphur atom and gold is very strong and the formed self-assembled  
monolayers (SAM's) are stable in air, water and organic solvents at room  
15 temperature [Bain, C. D.; Troughton, E. B.; Tao, Y.-T.; Evall, J.; Whitesides, G. M.;  
Nuzzo, R. G. *J. Am. Chem. Soc.* 1989, 111, 321-335]. It has been suggested that  
micro-contact printing [Mrksich, M.; Whitesides, G. M. *Tibtech* 1995, 13, 228-235]  
and photolithography [Bhatia, S. K.; Hickman, J. J.; Ligler, F. S. *J. Am. Chem. Soc.*  
1992, 114, 4432-4433] can be used to pattern surfaces with functionalized self-  
20 assembled monolayers for biosensor production with low cost for a diversity of  
applications, but until now it has not been possible to produce direct affinity sensors  
with high sensitivity.

#### Summary of the invention

25

It has now turned out that unexpectedly good metal ion-specific capacity affinity  
sensors suitable for determining the presence of a certain heavy metal ion by  
capacitance measurement, comprising the steps of:

- a) providing a piece of a noble metal, where said piece optionally can be a rod, or alternatively a piece of insulating material such as glass, silicon or quartz, on which a noble metal is sputtered or printed;
- b) providing a first SAM-forming molecule comprising a coupling group and/or a groups specifically binding to said heavy metal ion;
- c) contacting the piece in step a) with the first SAM-forming molecule in step b), thereby obtaining a self-assembling monolayer on said noble metal surface;
- d) in case the first SAM-forming molecule does not comprise a group specifically binding said heavy metal ion, contacting said self-assembling monolayer on said noble metal piece with a molecule specifically binding said heavy metal ion, thereby coupling said molecule to the self-assembling monolayer;
- e) contacting the piece obtained in step c) or d) with a second SAM-forming molecule, thereby obtaining a noble metal surface that is at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% covered with a self-assembling monolayer.

#### Detailed description of the invention

The detection limits reported in this invention are several orders of magnitude better than those reported previously for electrochemical metal detection methods. The insights behind this invention are that the recognition layer must be thin, well-ordered and it must cover at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% of the sensor surface. In a subsequent step, any free spots between the recognition elements are "plugged", i.e. covered with a second self-assembling monolayer-forming molecule, e.g. an alkanethiol comprising 3-25 carbon atoms preferably in a straight chain, after obtaining a self-assembling monolayer comprising affinity groups, thereby increasing the tightness and insulation. A capacitive biosensor is covered by an immobilized layer with the

recognition element toward the solution. Electrically it is equivalent to a capacitor between the conducting metal electrode and the conducting solution.

Any part of the surface that allows the aqueous solution to penetrate below the plane  
5 where the recognition takes place will act like a short-circuiting element. The capacitance will therefore increase due to the higher dielectric constant of the penetrating aqueous solution. Oxide layers are not well ordered and it is therefore impossible to form a dense recognition layer. Self-assembled monolayers are much better ordered and a more perfect coverage can therefore be expected in the  
10 immobilized layers. Furthermore, the self-assembled monolayers are much thinner than the oxide layers, resulting in a larger capacitance in series with the capacitance formed when molecules bind on the surface. This makes it easier to detect changes in the capacitance when an analyte binds to the surface.

15 This invention describes an affinity sensor for monitoring heavy metal ions at trace levels, optionally based on bioengineered proteins. A specifically heavy metal-binding substance is coupled to a conducting surface. The binding of the desired heavy metal ion to said substance causes a conformation change which is associated with a change in capacitance. The grafted recognition layer should be electrically  
20 insulating to prevent interferences from redox couples in the electrolyte and high Faradaic background currents. On the other hand, it should be as thin as possible in order to achieve high sensitivity. The use of self-assembled binding to gold or other self-assembling metals gives especially thin and compact layers. The invention also shows how additional insulation can be obtained by plugging with a different type of  
25 self-assembling molecule.

Accordingly, the present invention relates to a method for producing a metal ion specific capacity affinity sensor, wherein a piece of a noble metal is covered with a layer of a self-assembling monolayer comprising affinity groups. Subsequently any

free spots on the noble metal surface is covered by a second self-assembling monolayer-forming molecule.

In another aspect, the present invention relates to a metal ion specific capacity  
5 affinity sensor comprising a noble metal piece substantially completely covered with a self-assembling monolayer comprising affinity groups that specifically binds to a certain molecule of interest.

In yet another aspect, the present invention relates to a method for qualitatively or  
10 quantitatively determining the presence of a certain heavy metal ion of interest. A metal ion specific capacity affinity sensor, comprising a noble metal surface substantially completely covered with a self-assembling monolayer comprising affinity groups that specifically binds to a certain heavy metal ion of interest, is contacted with a liquid sample comprising the heavy metal ion of interest and the  
15 sensor's capacitance change is determined.

In a further aspect, the present invention relates to using said sensors for analysing certain heavy metal ions of interests, such as  $\text{Zn}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$ .

20 Definitions:

As disclosed herein, the terms "self-assembled monolayer" and "SAM" are  
synonyms and relate to the spontaneous adsorption of film components from a  
solution onto a solid surface making a well-ordered monolayer. Such a layer on gold  
25 substrates have previously been described [Porter, M.D.; Bright, T.B.; Allara, D.L.; Chidsey, C.E.D. *J. Am. Chem. Soc.* 1987, 109, 3559-3568].

As disclosed herein, the term "noble metal" relates to a metal chosen from the group of gold, silver, copper, platinum and palladium. Gold is preferred.

As disclosed herein, the terms "group/molecule specifically binding to a certain heavy metal ion of interest", respectively relate to a group or a molecule which specifically binds to a certain heavy metal ion. Any molecule having such binding characteristics can be used in the present invention. Examples of such molecules are chelating compounds and certain proteins. In a preferred embodiment, the proteins having the sequences according to anyone of SEQ.ID.NO. 1, SEQ.ID.NO.2, SEQ.ID.NO.3 and SEQ.ID.NO.4 are used.

As disclosed herein, the term SAM-forming molecule relates to a molecule having the ability of forming a self-assembling monolayer on a noble metal. A SAM-forming molecule comprises at least one thiol, sulphide or disulphide group and may optionally also comprise a heavy metal-binding group. When a suitable heavy metal-binding molecule, e.g. a protein or a peptide, comprises a thiol, sulphide or disulphide group, it might be a SAM-forming molecule per se. Most heavy metal-binding molecules, are, however, not SAM-forming molecules on their own. They must be coupled to small SAM-forming molecules in a separate step. Examples of such small SAM-forming molecules are thioctic acid and cysteamine. This coupling step is carried out after formation of the self-assembling monolayer on the noble metal surface. The skilled person is well aware of how to choose suitable coupling reactions and coupling groups. In the following examples, a self-assembling monolayer consisting of thioctic acid is activated by 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide. Subsequently a heavy metal-binding molecule is coupled to the activated monolayer. However, other similar coupling reactions are described in the literature.

As disclosed herein, the term "plugging" refers to treatment in a solution containing a thiol, sulphide or disulphide after immobilization of the affinity group either to a self-assembling monolayer on a noble metal surface or, in case the affinity molecule

comprises available thiol, sulphide or disulphide groups rendering the affinity molecule to be a SAM-forming molecule per se, directly to the noble metal surface, in order to block any unblocked spots on said surface. As already mentioned, it is necessary that the noble metal surface is as completely covered by a SAM as possible in order to optimize the sensitivity of the sensor.

As disclosed herein, the term "heavy metal ion" relates to metal ions having atomic numbers higher than 21, such as ions of Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Zr, Nb, Mo, Ru, , Rh, Pd, Ag, Cd, In, Sn, Ta, W, Re, Os, Ir, Pt, Au, Hg, Tl, Pb and Bi. The present invention is particularly usefull for determining the presence of  $\text{Zn}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$ .

As disclosed herein, the term "functional derivatives" relates to derivatives in which the original amino acid sequence has been modified or changed by insertion, addition, substitution or exclusion of one or more amino acids, as well as derivatives containing multiples of the original sequence or parts thereof.

As disclosed herein, SCE stands for the saturated calomel electrode; Potentiostatic perturbation means a fast change in potential. PEGDGE stands for polyethylene-glycol-di-glycidyl-ether. BSA means bovine serum albumin.

In this invention a solution containing the heavy metal ions to be determined is allowed to have electrical contact with a conducting surface containing the recognition element after which the capacitance or impedance change is determined . The capacitance change takes place between the solution and a metal surface, consisting of solid metal or metal sputtered on an underlying non-conducting surface. Faradaic reactions with the metal as well as background currents are blocked by the recognition element on the surface, eventually improved by treatment with auxiliary compounds which improve the insulation. The recognition element is



bound to the metal surface, either directly through self-assembly, or by binding it to a self-assembled compound on the electrode. It can also be bound through adsorption, polymerization or coating. Measurements are made by applying varying voltages to the electrode using potentiostatic methods and analyzing the current changes. The sensitivity can be improved by allowing a solution containing a secondary specific ligand to bind to the analyte already on the surface, thereby increasing the size of the bound aggregate and the capacitance change.

The invention will now be described in more detail with reference to the enclosed drawings.

Fig. 1 shows schematically how a heavy metal specific protein is immobilized on the metal surface and how its three-dimensional conformation is changed when heavy metal ions bind to the protein.

Fig. 2 shows the measuring flow cell, a) measuring electrode, b) auxiliary platinum foil electrode, c) platinum wire reference electrode, d) Ag/AgCl reference electrode.

Fig. 3 shows the response for copper for electrodes immobilized with GST-*SmtA* protein with EDC-mediated coupling (upper curve) and PEGDGE entrapment (lower curve). See example 1 for more details.

Fig. 4 shows the cyclic voltammetry responses recorded in a  $\text{Fe}(\text{CN})_6^{3-}$  solution when the measuring electrode was a) unmodified gold, b) gold modified with thioctic acid, c) as in b with additional modification with immobilized fusion protein GST-*SmtA* and d) as in c with additional modification with 1-dodecanethiol. More details are given in example 2.

Fig. 5 shows capacitance change *versus* heavy metal ion concentration for a GST-*SmtA* immobilized electrode. Curve 1) shows the response for  $\text{Zn}^{2+}$ , curve 2) for  $\text{Hg}^{2+}$ , curve 3) for  $\text{Cd}^{2+}$  and curve 4) for  $\text{Cu}^{2+}$ . The measurements were performed in 100 mM borate buffer, pH 8.75 at a flow rate of 0.5 ml/min. Samples with a volume of 250  $\mu\text{l}$  were injected. For more details see example 3.

Fig. 6 shows capacitance change *versus*  $\text{Cu}^{2+}$  concentration for electrodes using EDC coupling of protein. Upper curve shows the signal for the GST-*SmtA*-based electrode and the lower curve represents the signal recorded by a blank electrode with BSA on the surface. The measurements were performed in 100 mM borate buffer, pH 8.75 at a flow rate of 0.5 ml/min. Samples with a volume of 250  $\mu\text{l}$  were injected. More details are given in example 3.

Fig. 7 shows capacitance change *versus*  $\text{Cu}^{2+}$  concentration in the range  $10^{-15}$  M to 0.1 M. The measurements were performed in 100 mM borate buffer, pH 8.75 and a flow rate of 0.5 ml/min. Samples with a volume of 250  $\mu\text{l}$  were injected. For details see example 3.

Fig. 8 shows a stability study for a GST-*SmtA* electrode according to the present invention. A calibration curve from 1 femtomolar to 100 picomolar was recorded each day and the total capacitance change vs. time was plotted. Between measurements the sensor was stored at 4°C in 100 mM borate buffer pH 8.75. Details are given in example 3.

Fig. 9 shows capacitance change *versus* heavy metal ion concentration for a *MerR* immobilized electrode. Curve 1) shows the response for  $\text{Cd}^{2+}$ , curve 2) for  $\text{Cu}^{2+}$  and curve 3) for  $\text{Hg}^{2+}$ . The measurements were performed in 100 mM borate buffer, pH 8.75 and a flow rate of 0.5 ml/min. Samples with a volume of 250  $\mu\text{l}$  were injected. More details are given in example 4.

If a solid measuring metal electrode is used, a gold rod of typically 3 mm in diameter, is polished, cleaned and coated through self-assembly with a recognition element or with a compound which can be coupled with a recognition element. A  
5 great number of coupling methods are known and may be used as suitable alternatives to those described in the examples. It is also possible to use metal sputtered or printed on glass, quartz, silicon or another insulating material as disposable electrodes. After cleaning the electrodes are coated in batch and inserted in a quick-connect measuring cell. A number of different recognition elements can  
10 be immobilized on the same sputtered electrode if they are separated by insulating parts and connected to the potentiostat with switches which can be controlled by a microprocessor.

The inverse total capacitance is the sum of the inverse capacitances of each layer in series, i. e. the thioctic acid layer, the protein layer and the capacitance between the  
15 double layer space charge and solution. If one of these is small compared to the others, it will dominate the total capacitance. Especially if self-assembled parts give rise to a small capacitance, it will dominate over the capacitances in the recognition layer. Changes in the recognition layer will thus have little effect on the total  
20 capacitance resulting in a low overall sensitivity of the sensor.

The electrode is inserted into a cell which may be either of the cuvette type or a flow cell as shown in Fig. 2. The cell must contain an auxiliary electrode, typically a platinum foil which should be placed symmetrically and opposite to the measuring  
25 electrode. A reference electrode, typically SCE or Ag/AgCl, is placed in the cell so that the voltage drop between the reference and measuring electrodes due to capacitive or Faradaic currents becomes very small. In some cases the performance may be improved if a very small additional reference electrode is used, see Fig. 1c and the SCE reference is moved away, Fig. 1d. A flow cell gives more precise

control over the mass transfer to the measuring electrode and injection of sample and cleaning up is more easily automated. Flow cells with volumes of 2 ml and 10  $\mu$ l were found to have about the same sensitivity. A flow cell with disposable electrodes made by sputtering gold on silicon also had similar properties.

5

The electrodes are connected to a fast potentiostat which in turn is controlled by a microprocessor. The potentiostat will keep the measuring electrode at a pre-set value *versus* the reference electrode. A potentiostatic perturbation is imposed on the measuring electrode. It may be a sinusoidal wave, it may be a step voltage or any  
10 other wave-form which enables interpretation of the results. The currents caused by the perturbation voltage are used for evaluation of the capacitance of the measuring electrode. It can be done for sinusoidal perturbations by separating the in-phase and out-phase part of the current in a lock-in amplifier. If this is repeated at different frequencies an impedance spectra can be obtained. In all cases the capacitance can  
15 be evaluated from the current responses. As usual in electrochemistry the role of current and potential can be reversed, i. e. a galvanostatic step in current will cause the potential to change. This change can be used for evaluation of the capacitance, although using different algorithms.

20 A known volume of sample is normally mixed with a known volume of a conducting liquid in e.g. a cuvette in a batch type cell. For a flow cell, a known volume is injected into a conducting carrier flow pumped with a distinct flow rate. The conducting liquids are normally buffers with ionic strengths from a few millimolar and up. The sample can be in a non-conducting medium but a conducting solution  
25 must fill the cell when measurements are made.

The present invention will now be further described with reference to the following examples. The examples are given for the purpose of information and are not intended to limit the scope of the present invention.

### Example 1.

The fusion proteins GST-*SmtA* and *MerR* were produced as described below and dissolved in phosphate buffered saline ( 70 mM NaCl, 1.3 mM KCl, 5 mM  
5 Na<sub>2</sub>HPO<sub>4</sub>, 0.9 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) containing 50 % (v/v) glycerol to a final concentration of 1 mg/ml protein. Thiocetic acid and glutaraldehyde were purchased from Sigma and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride was obtained from Fluka. 1-dodecanethiol and the gold rods used for the electrodes (Cat. No 26,583-7, = 3 mm) came from Aldrich Chemicals. The heavy metals CuCl<sub>2</sub>  
10 \*2H<sub>2</sub>O, ZnCl<sub>2</sub>, HgCl<sub>2</sub> and Cd(NO<sub>3</sub>)<sub>2</sub>\*4H<sub>2</sub>O were all from Merck (Darmstadt, Germany). PEGDGE was achieved from Polysciences Inc. (USA). All other reagents used were of analytical grade.

The biosensors were prepared by immobilizing fusion proteins on the gold surface  
15 by EDC-mediated coupling, PEGDGE entrapment or glutaraldehyde coupling. In all cases 20 µl of the dissolved fusion proteins were diluted with 480 µl 100 mM borate buffer, pH 8.75 and the solution was ultrafiltered on a micro-filter (Amicon, USA) with a molecular cut-off of 3000 D. After the ultrafiltration the filter was turned over to recover fusion protein and its concentration was adjusted to 0.04 mg/ml in borate  
20 buffer. Gold electrodes were cleaned and pretreated with self-assembling of thiocetic acid, as described earlier by Berggren and Johansson [Berggren, C., Johansson, G., *Anal. Chem.*, Submitted ]. As the *MerR* protein is sensitive against oxidation in air, the preparation of these electrodes was performed under nitrogen atmosphere.

25 *EDC-mediated coupling:* The self-assembled electrodes were thoroughly washed with ethanol, dried and thereafter activated in a 1 % solution of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride in dried acetonitrile for 5 hours. After washing with 100 mM borate buffer, pH 8.75 the electrodes were dipped into the protein solution at a temperature of 4°C for 24 hours. Subsequently

each electrode was thoroughly washed with borate buffer and dipped into a solution of 1-dodecanethiol for 20 minutes.

*PEGDGE entrapment:* The thioctic acid activated electrode was covered with  
5 1.5  $\mu$ l of a 0.04 mg/ml protein solution in 100 mM borate buffer containing 30 % (w/v) PEGDGE and the electrode was incubated at 45°C for 15 minutes.

Afterwards the electrode was treated with 1-dodecanethiol for 20 minutes, washed with borate buffer and placed in the measuring cell.

10 *Glutaraldehyde coupling:* Prior to glutaraldehyde immobilization the electrode was modified with cysteamine, instead of thioctic acid. The dried electrode was dipped into a solution containing 12.5 % (w/v) glutaraldehyde in coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7) and 6 g/l NaCNBH<sub>3</sub> for 4 hours. Then, the electrode was washed thoroughly with coupling buffer and dipped into the protein  
15 solution (0.04 mg/ml) with 6 g/l NaCNBH<sub>3</sub> for 4 hours. Finally the electrode was washed thoroughly with coupling buffer before use.

*Capacitance measurements:* The biosensor was arranged as the working electrode in a three-electrode system connected to a fast potentiostat, as shown in figure 1. It was  
20 placed in a flow cell with a dead volume of 10  $\mu$ l which was built in house. A platinum foil served as the auxiliary and a platinum wire as the reference electrode. An extra reference electrode (Ag/AgCl) was placed in the outlet stream, as the platinum does not have a defined potential. The buffer solution was pumped by a peristaltic pump with a flow rate of 0.5 ml/min. through the flow cell. Samples were  
25 injected into the flow *via* a 250  $\mu$ l sample loop. The buffer used was 10 mM borate, pH 8.75. Before use it was filtered through a 0.22  $\mu$ m millipore filter and degassed.

The working electrode had a rest potential of 0 mV vs. the Ag/AgCl reference electrode. When measuring, a potential step of 50 mV was applied and the current

transient that followed when the potential increased was assumed to follow the equation:

$$i(t)=u/R_s \exp(-t/R_s * C_1) \quad (1)$$

5

where  $i(t)$  is the current at time  $t$ ,  $u$  is the amplitude of the potential pulse applied,  $R$  is the resistance between the gold electrode and the reference electrode,  $C_1$  is the total capacitance over the immobilized layer and  $t$  is the time elapsed after the potential pulse was applied. The current values were collected with a frequency of 50 kHz and the first ten values were used in the evaluation of the capacitance. Each capacitance value was calculated as the mean of ten measurements.

10

With capacitance measurements the interface between a conducting electrode and the sample solution is studied. This interface is called the electrical double-layer as charges and dipoles will orient with opposite signs on the conducting surface and in the solution. When the heavy metal specific protein is immobilized on the electrode surface it will exchange the buffer solution at the surface and a charge separation will take place, leading to a decrease in capacitance. The detection principle used in this work is based on monitoring a conformational change resulting from a specific binding of a certain heavy metal ion to an immobilized protein on the electrode surface (see Fig. 2). As the sensor measures changes in conformation, it is important to optimize the immobilization conditions.

15

20

25

*Immobilization of the recognition element:* A gold electrode was used as conducting noble metal surface in this work. To immobilize the recognition elements, which were different fusion proteins, the gold surface had to be modified in some way. A well-known and easy method is to use self-assembling of thiol-, sulphide- or disulphide compounds onto the gold surface. Self-assembling is a spontaneous process producing well-ordered monolayers of molecules. Furthermore the binding

between sulphur and gold is rather strong, an important stability aspect in biosensor design, where leaking off of the recognition element results in loss of activity. Three different immobilization methods were studied in order to produce an optimal surface for capacitance measurements, namely; EDC-mediated coupling, PEGDGE entrapment and glutaraldehyde coupling. In all cases a short compound containing one or more sulphur groups was first self-assembled on the gold surface. The EDC-mediated coupling is producing a monolayer of protein molecules on the surface without any crosslinking between individual molecules. This method has previously been shown to be successful for immobilization of antibodies onto gold electrodes with self-assembled thioctic acid on the surface [Duan, C.; Meyerhoff, M. E., *Anal. Chem.*, 66, 1994, 1369-1377]. With PEGDGE immobilization the protein will be entrapped in a polymer network, where the heavy metals can diffuse into the pores of the polymer. Calibration curves for copper are shown in figure 3 for EDC coupling (upper curve) and for PEGDGE entrapment (lower curve) of the protein. It was found that the sensitivity was much higher for the EDC activated electrode. A possible explanation might be that the PEGDGE entrapment prohibits the conformational change of the fusion protein to some extent. The reproducibility was also lower for the PEGDGE electrodes, because of the difficulty to deposit exactly the same amount of the protein/crosslinker for each electrode. Further experiments will be performed with less concentration of PEGDGE to decrease the crosslinking and thereby produce a less rigid protein layer. Glutaraldehyde is also a crosslinker, but in this case the surface was first activated by glutaraldehyde before the protein was added. Also in this case it is very important to optimize the amount of crosslinker in order not to crosslink the protein too much, making it rigid and not susceptible to conformational changes. All further experiments were performed with electrodes manufactured by the EDC method.



### Example 2

Cyclic voltammograms were recorded in 5 mM  $K_3[Fe(CN)_6]$ , 1 M KCl in a batch cell, with the unmodified/modified gold electrode as working electrode a SCE as  
5 reference and a platinum flag as auxiliary electrode. The scan rate was 50 mV/s. The electrodes were connected to a Princeton Applied 274 A potentiostat, connected to a computer.

In order to develop a sensitive affinity sensor based on capacitance measurements  
10 the immobilization layer has to be as thin as possible and well-ordered. Defect sites in the monolayer might lead to penetration of solution molecules through the layer and close to the metal surface, leading to short-circuiting of the recognition layer with highly conducting aqueous channels. The result will be that capacitance changes will be smaller or absent for such defect sensors. The importance of good  
15 insulation of the electrode surface has earlier been demonstrated [Berggren, C.; Johansson, G. *Anal. Chem.* Submitted]. The degree of insulation can be studied by having a small permeable redox couple in solution. In figure 4 it is shown how the blocking increases for each additional layer. For a clean gold surface the redox couple  $K_3[Fe(CN)_6]$  is oxidized and reduced at the metal surface. A surface covered  
20 with self-assembled thioctic acid reduces the access to the surface. Immobilization of the protein further insulates the surface, but it is not until the treatment in 1-dodecanethiol that the oxidation/reduction peaks totally disappears. All electrodes in the following experiments were therefore treated twenty minutes with 1-dodecanethiol.

### Example 3

Characteristics such as sensitivity, selectivity, response time, stability and electrode surface regeneration possibility were studied in this work. However, the GST-SmtA

based electrodes were more thoroughly studied, but some characteristics were studied for MerR protein based electrodes as well, and obtained results were evaluated and compared. The affinity of the GST-SmtA protein was studied for copper, cadmium, mercury and zinc (see Fig. 5). The electrodes responded for all  
5 four metal ions with decreasing sensitivity for  $\text{Cu} > \text{Cd} > \text{Hg} > \text{Zn}$ , well in agreement with previously reported results obtained with solution of this engineered protein [Shi, J.; Lindsay, W. P.; Huckle, J. W.; Morby, A. P.; Robinson, N. J. *FEBS*, 303, 1992 159-163]. The detection limit was in the femtomolar molar range or below for each of the studied heavy metals. Reference electrodes, equally prepared but  
10 using BSA instead of GST-SmtA resulted in hardly detectable signals for Cu and no signals at all for the other ions (see Fig. 6). Similar results were obtained when using another protein, urease, well known to bind heavy metals. For the latter case, the absence of any detectable signals was assumed to be due to lack of conformational changes upon exposure to heavy metal.

15

The activity of an GST-SmtA electrode was studied for an extended interval. Copper samples of concentrations of  $10^{-15}\text{M}$  to  $0.1\text{ M}$  were injected and the capacitance changes were determined. A slow increase of the signal was obtained to approximately  $10^{-5}\text{M}$  thereafter the signal increase became much faster to  
20 approximately  $10^{-2}\text{M}$ , where saturation occurred (see Fig. 7). It is believed that the increase in the first part of the curve is due to the specific binding of heavy metal and that the second one probably is due to denaturation of the protein on the electrode surface. Inability to regenerate the electrode after injection of a  $1\text{mM}$  copper might support this assumption. The stability of the GST-SmtA based  
25 biosensor was studied for 16 days. A calibration curve between  $10^{-15}\text{M}$  to  $10^{-10}\text{M}$  Cu was measured each day and the total capacitance change vs. time is plotted in figure 8. Between measurements the sensor was stored at  $4^{\circ}\text{C}$  in  $100\text{ mM}$  borate buffer, pH 8.75. A loss of activity was observed after 10 days and it decreased with approximately 40% after 16 days.

The surface of the biosensor could be regenerated by injecting 250Tl, 1 mM EDTA. It was found that if EDTA was injected just before storage the biosensor lost activity over night, but if the electrode was stored with heavy metal and the  
5 regeneration procedure took place immediately before measuring the next day no activity loss could be observed. The observed phenomenon was attributed to the assumption that the protein has a more open structure when the heavy metal ion is absent and hence this structure is more easily denatured. Regeneration with EDTA after injection of a sample containing as much as 1 mM  $\text{Cu}^{2+}$  was unsuccessful,  
10 attributed to irreversible surface denaturation by this high metal ion concentration.

#### Example 4

Biosensors were constructed using the second engineered protein, MerR, and signals  
15 were recorded for Cu, Cd, Hg and Zn. This protein was reported to be highly specific towards mercury (II) ions [Frantz, B.; O'Hallaran, T. V.; *Biochemistry*, 29, 1990 4747-4751]. The protein was immobilized onto the gold surface only by using the EDC-coupling method. Obtained results were in good agreement with reported properties, the MerR-based electrodes displayed highest selectivity for mercury and  
20 that this electrode showed higher selectivity than the GST-SmtA based electrodes (see Fig. 9).

Ala Cys Ser Gly Ala Arg Pro Ala Gln Ser Cys Gly Ile Leu Gln Gly  
 115 120 125

Leu Ser Asp Cys Val Cys Asp Thr Arg Gly Thr Thr Ala His Pro Ser  
 130 135 140

Asp  
 145

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 72 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Pseudomonas aeruginosa*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Thr Gln Thr Val Thr Leu Ser Val Pro Gly Met Thr Cys Ser Ala  
 1 5 10 15

Cys Pro Ile Thr Val Lys Lys Ala Ile Ser Glu Val Glu Gly Val Ser  
 20 25 30

Lys Val Asp Val Thr Phe Glu Thr Arg Gln Ala Val Val Thr Phe Asp  
 35 40 45

Asp Ala Lys Thr Ser Val Gln Lys Leu Thr Lys Ala Thr Ala Asp Ala  
 50 55 60

Gly Tyr Pro Ser Ser Val Lys Gln  
 65 70

Leu Val Cys Ala Cys His Ala Arg Arg Gly Asn Val Ser Cys Pro Leu  
 115 120 125

Ile Ala Ser Leu Gln Gly Gly Ala Ser Leu Ala Gly Ser Ala Met Pro  
 130 135 140

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 145 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Alcaligenes eutrophus*

(B) STRAIN: CH34

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Asn Ile Gln Ile Gly Glu Leu Ala Lys Arg Thr Ala Cys Pro Val  
 1 5 10 15

Val Thr Ile Arg Phe Tyr Glu Gln Glu Gly Leu Leu Pro Pro Pro Gly  
 20 25 30

Arg Ser Arg Gly Asn Phe Arg Leu Tyr Gly Glu Glu His Val Glu Arg  
 35 40 45

Leu Gln Phe Ile Arg His Cys Arg Ser Leu Asp Met Pro Leu Ser Asp  
 50 55 60

Val Arg Thr Leu Leu Ser Tyr Arg Lys Arg Pro Asp Gln Asp Cys Gly  
 65 70 75 80

Glu Val Asn Met Leu Leu Asp Glu His Ile Arg Gln Val Glu Ser Arg  
 85 90 95

Ile Gly Ala Leu Leu Glu Leu Lys His His Leu Val Glu Leu Arg Glu  
 100 105 110

Leu Tyr Tyr Cys Ser Glu Ala Cys Ala Asp Gly His Thr Gly Gly Ser  
 260 265 270

Lys Gly Cys Gly His Thr Gly Cys Asn Cys Ser Glu Phe Ile Val Thr  
 275 280 285

Asp

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 144 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Pseudomonas aeruginosa*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Glu Asn Asn Leu Glu Asn Leu Thr Ile Gly Val Phe Ala Lys Ala  
 1 5 10 15

Ala Gly Val Asn Val Glu Thr Ile Arg Phe Tyr Gln Arg Lys Gly Leu  
 20 25 30

Leu Leu Glu Pro Asp Lys Pro Tyr Gly Ser Ile Arg Arg Tyr Gly Glu  
 35 40 45

Ala Asp Val Thr Arg Val Arg Phe Val Lys Ser Ala Gln Arg Leu Gly  
 50 55 60

Phe Ser Leu Asp Glu Ile Ala Glu Leu Leu Arg Leu Glu Asp Gly Thr  
 65 70 75 80

His Cys Glu Glu Ala Ser Ser Leu Ala Glu His Lys Leu Lys Asp Val  
 85 90 95

Arg Glu Lys Met Ala Asp Leu Ala Arg Met Glu Ala Val Leu Ser Glu  
 100 105 110

(A) ORGANISM: *Synechococcus* sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro
1      5      10      15

Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
20     25     30

Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
35     40     45

Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
50     55     60

Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
65     70     75     80

Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
85     90     95

Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
100    105    110

Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
115    120    125

Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
130    135    140

Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
145    150    155    160

Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
165    170    175

Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
180    185    190

Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
195    200    205

Thr Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Ile Glu Gly
210    215    220

Arg Gly Ile Pro Met Thr Ser Thr Thr Leu Val Lys Cys Ala Cys Glu
225    230    235    240

Pro Cys Leu Cys Asn Val Asp Pro Ser Lys Ala Ile Asp Arg Asn Gly
245    250    255

```

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Vlaamse Instelling voor Technologisch  
Onderzoek

(B) STREET: Boeretang 200

(C) CITY: Mol

(E) COUNTRY: Belgium

(F) POSTAL CODE (ZIP): 2400

(G) TELEPHONE: + 32 14 33 51 12

(H) TELEFAX: +32 14 32 03 72

(A) NAME: The University of Birmingham

(B) STREET: Edgbaston

(C) CITY: Birmingham

(E) COUNTRY: United Kingdom

(F) POSTAL CODE (ZIP): B15 2TT

(A) NAME: Lund University

(B) STREET: P.O. BOX 124

(C) CITY: Lund

(E) COUNTRY: Sweden

(F) POSTAL CODE (ZIP): 221 00

(ii) TITLE OF INVENTION: Metal ion-specific capacity affinity sensor

(iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 289 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:



### Claims

1. A method for producing a metal ion-specific capacity affinity sensor suitable for determining the presence of a certain heavy metal ion by capacitance measurement,  
5 comprising the steps of:
  - a) providing a piece of a noble metal, where said piece optionally can be a rod, or alternatively a piece of insulating material such as glass, silicon or quartz, on which a noble metal is sputtered or printed;
  - b) providing a first SAM-forming molecule comprising a coupling group and/or a  
10 group specifically binding to said heavy metal ion;
  - c) contacting the piece in step a) with the first SAM-forming molecule in step b), thereby obtaining a self-assembling monolayer on said noble metal surface;
  - d) in case the first SAM-forming molecule does not comprise a group specifically binding said heavy metal ion, contacting said self-assembling monolayer on said  
15 noble metal piece with a molecule specifically binding said heavy metal ion, thereby coupling said molecule to the self-assembling monolayer;
  - e) contacting the piece obtained in step c) or d) with a second SAM-forming molecule, thereby obtaining a noble metal surface that is at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99%  
20 covered with a self-assembling monolayer.
2. A method according to claim 1, characterized in that the coupling reaction in step d) is carried out in presence of PEGDGE.
- 25 3. A method according to claim 1, characterized in that the piece is exposed to a solution containing a crosslinking substance such as glutaraldehyde prior to step d).
4. A method according to claim 1, characterized in that the first SAM-forming molecule is D/L-thioctic acid, and in that said D/L-thioctic acid is activated with 1-  
30 (3-dimethylaminopropyl)-3-ethyl-carbodiimide before step d) is carried out.

5. A method according to claim 1, characterized in that the second SAM-forming molecule is a thiol comprising 3-25 carbon atoms in a straight saturated chain, and preferably is 1-dodecanethiol.

5 6. A metal ion-specific capacity affinity sensor comprising a piece of a noble metal, where said piece optionally can be a rod, or alternatively a piece of insulating material such as glass, silicon or quartz, on which a noble metal is sputtered, to which piece groups specifically binding to a certain heavy metal ion of interest have been bound characterized in that said groups specifically binding to said heavy  
10 metal ion are comprised in a self-assembling monolayer covering at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% of the noble metal surface.

7. A metal ion capacity affinity sensor according to claim 6, characterized in that  
15 said sensor has been produced by a method according to anyone of claims 1-6.

8. A sensor according to claim 6, characterized in that specifically heavy metal ion-binding groups are selected from the group of proteins having the sequences SEQ.ID.NO.1, SEQ.ID.NO.2, SEQ.ID.NO.3 or SEQ.ID.NO.4, or functional  
20 derivatives thereof having equivalent binding characteristics.

9. A method for qualitatively or quantitatively determining the presence of a certain heavy metal ion of interest in a liquid sample, comprising the steps of:

- 25 a) providing a sensor according to claim 6, wherein said affinity groups specifically binds to said heavy metal ion of interest;
- b) contacting said sensor with a reference liquid not containing said heavy metal ion of interest and determining the capacitance according to per se known methods;
- c) contacting said sensor with a sample suspected of containing said heavy metal ion and determining the capacitance according to per se known methods; and

d) calculating the difference between the capacitance of the sample and the capacitance of the reference sample, and optionally calculating the amount of said compound by using prerecorded calibration data.

5 10. A method according to claim 9 for determining the presence of ions selected from the group of  $\text{Zn}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Pb}^{2+}$ .

11. Use of a sensor according to claim 6 for determining the presence of of ions selected from the group of  $\text{Zn}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$ .

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**Abstract**

This invention relates to a metal ion-specific capacitance sensor with exceptional sensitivity and wide operating range. It is versatile because different kinds of recognition elements can be immobilized directly in a self-assembling monolayer substantially completely covering the surface of the measuring noble metal electrode. The electrode then becomes selective to those metal ions in the solution that show affinity to the recognition element on the surface. Compared to previously described electrochemical sensors, the sensor according to the present invention shows many orders of magnitude better sensitivity because of the unique measuring principle.

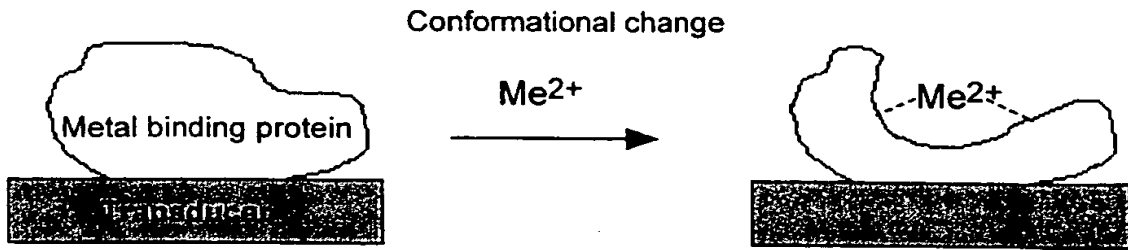


Fig. 1

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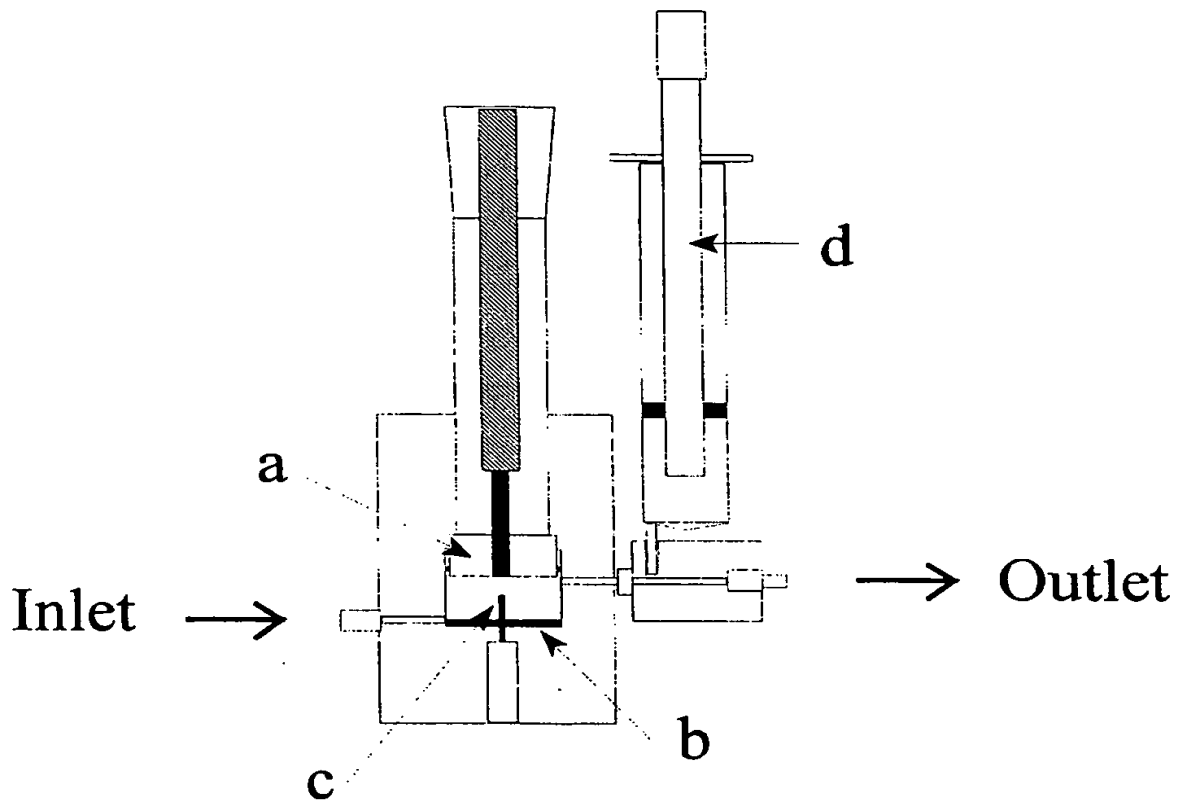
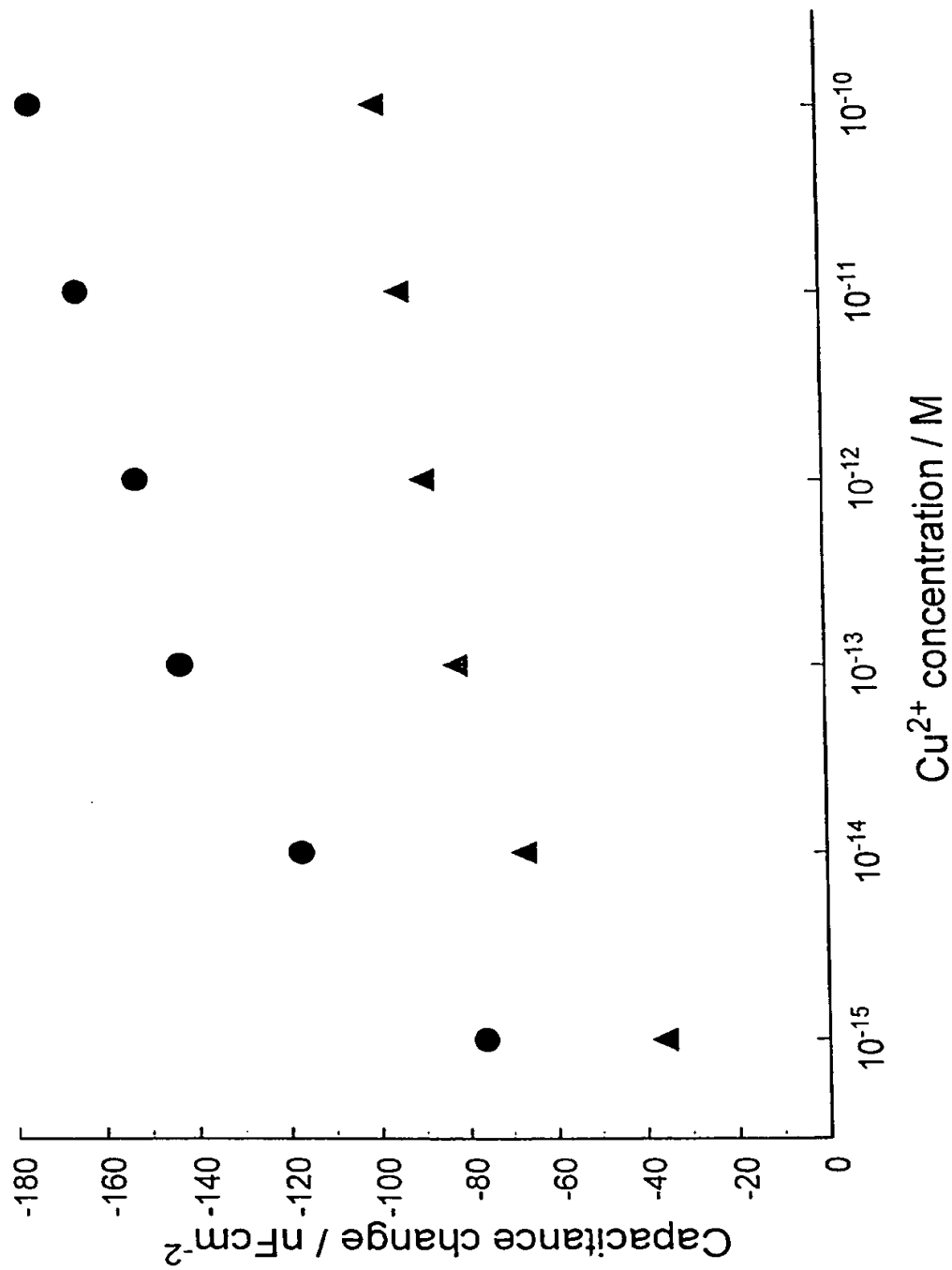
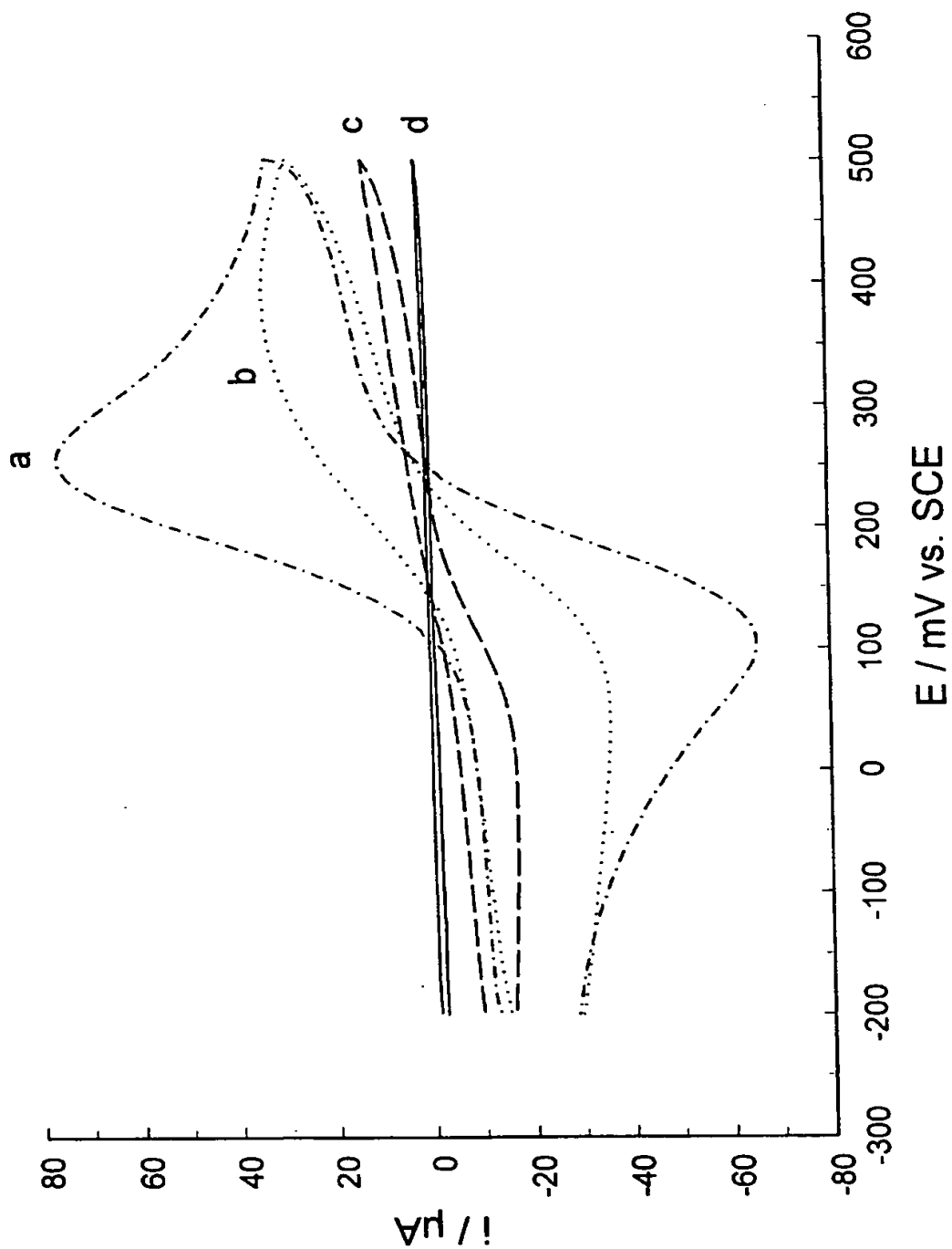
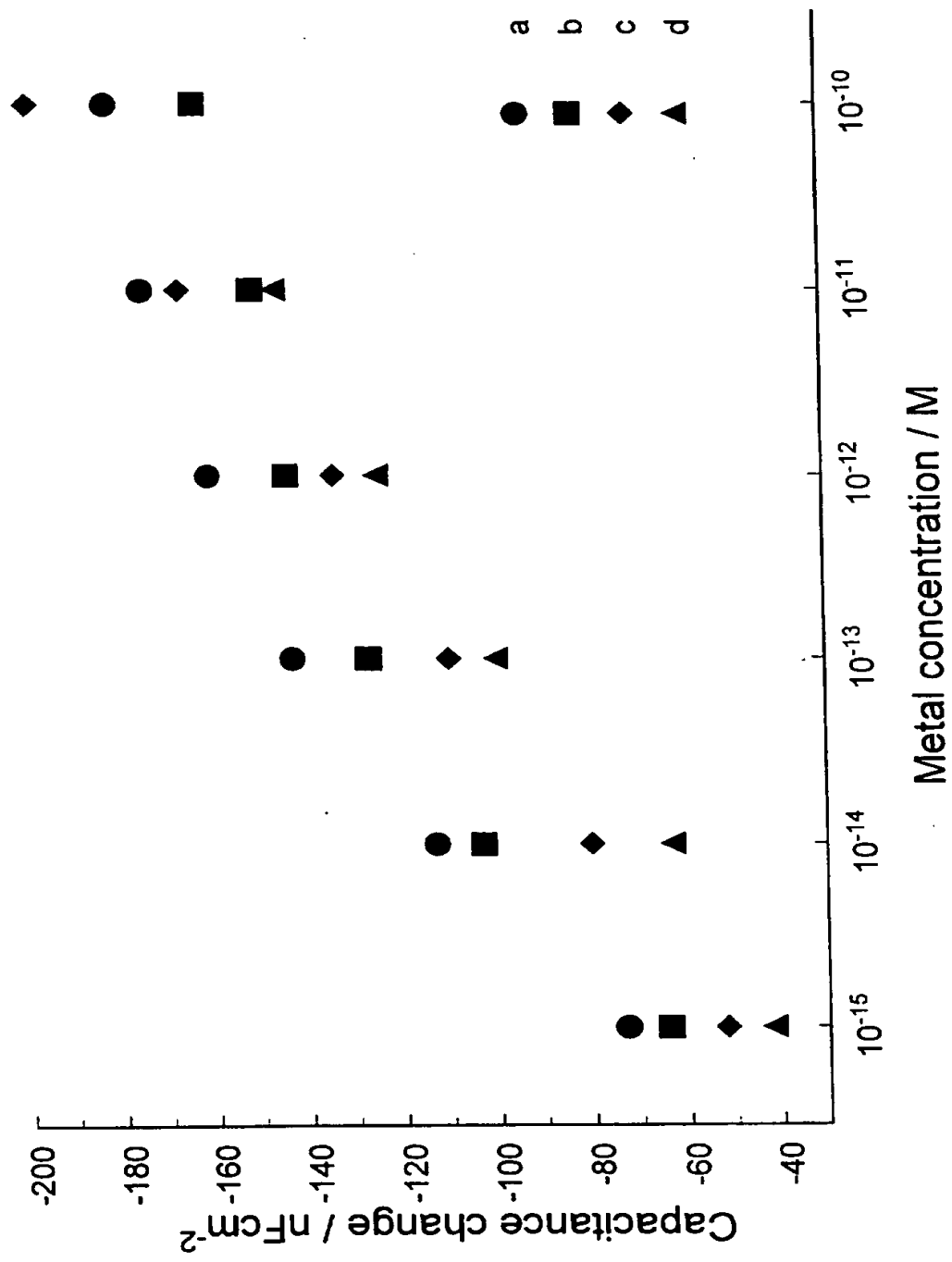


Fig. 2









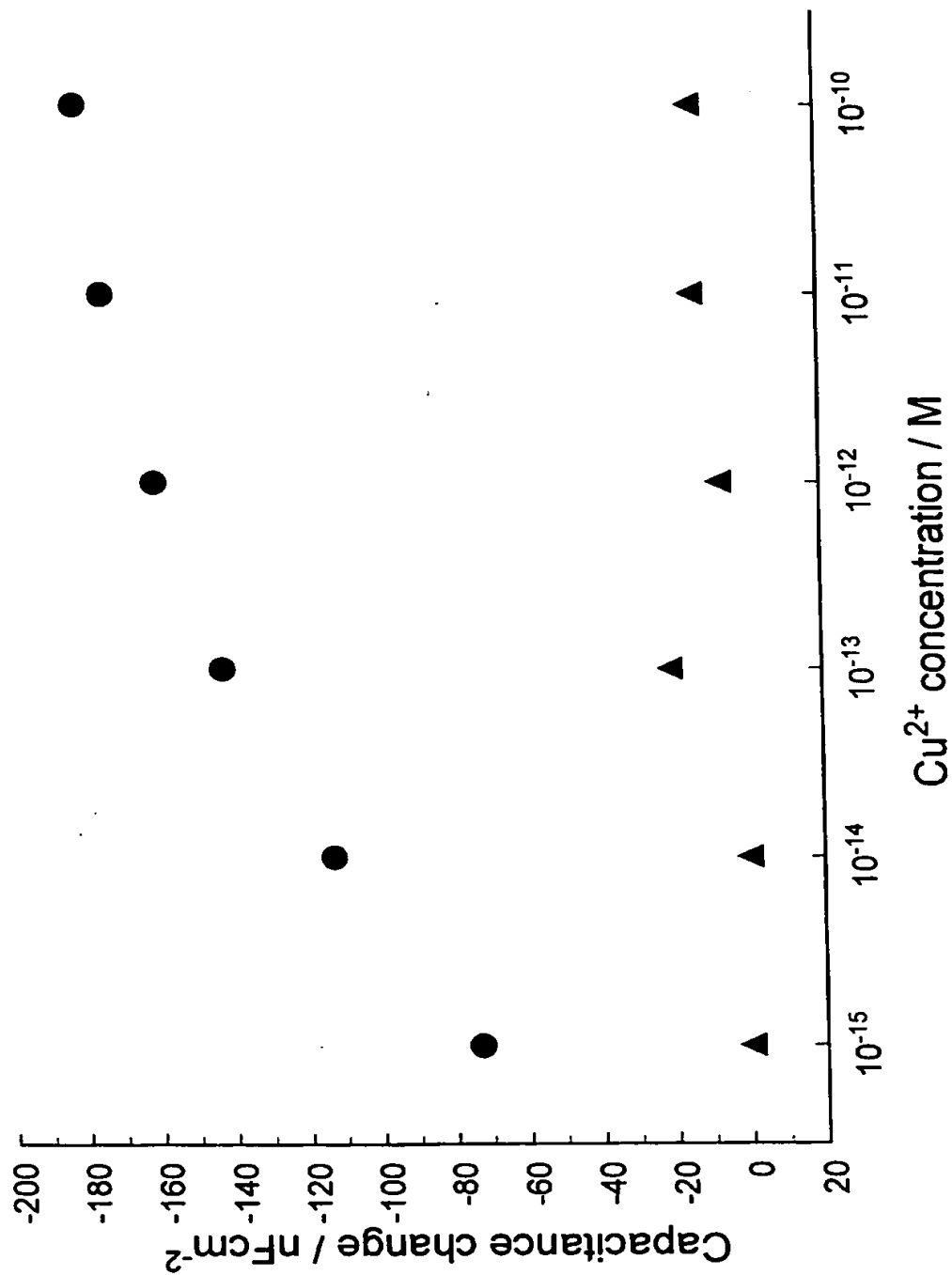
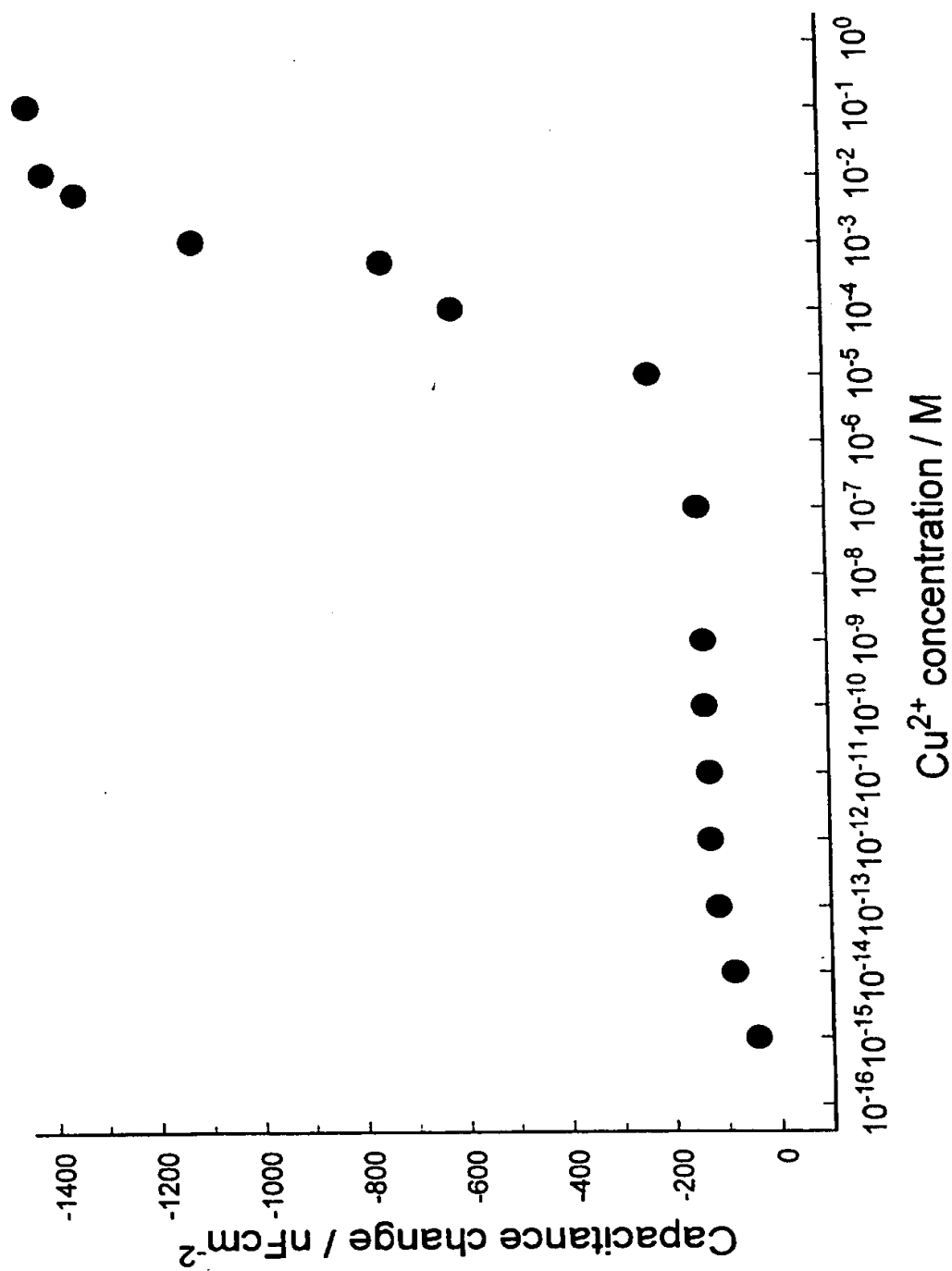
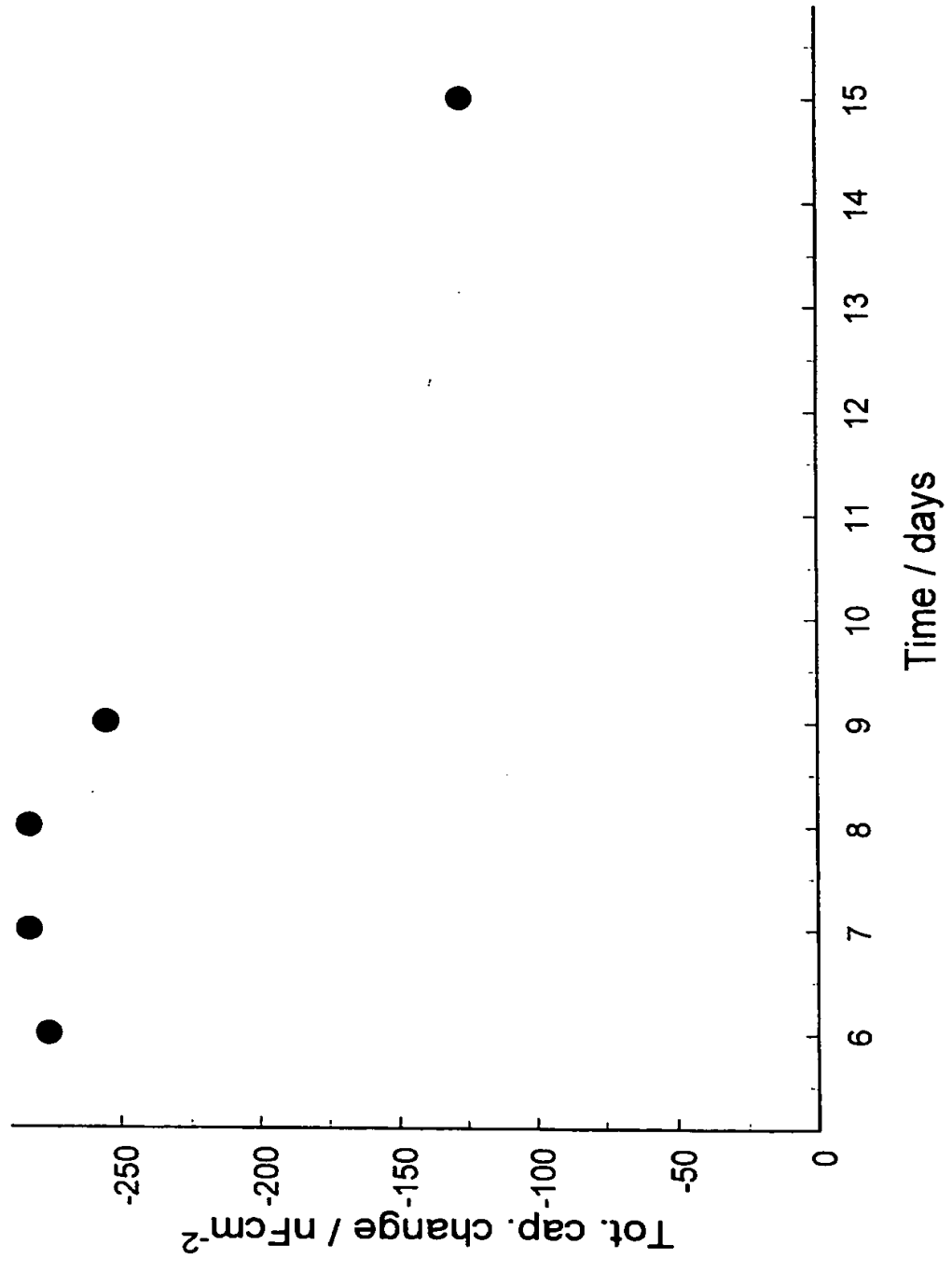


Fig. 7



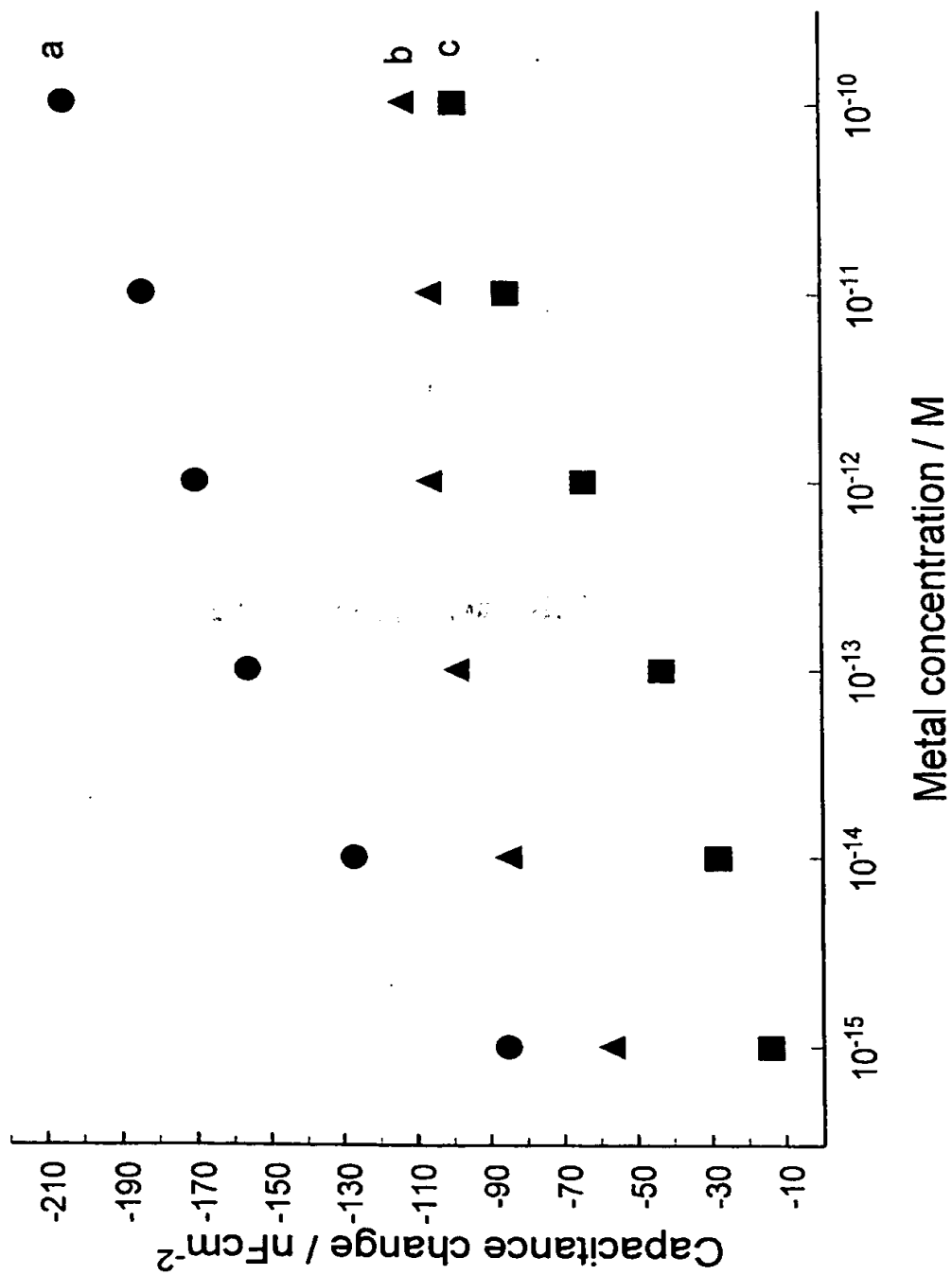
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